

Isolation of human monoclonal autoantibodies derived from pancreatic lymph node and peripheral blood B cells of islet autoantibody-positive patients

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Abstract

Aims/hypothesis Autoantibodies against pancreatic islets and infections by enteroviruses are associated with type 1 diabetes, but the specificity of immune responses within the type 1 diabetic pancreas is poorly characterised. We investigated whether pancreatic lymph nodes could provide a source of antigen-specific B cells for analysis of immune responses within the (pre)diabetic pancreas.

Methods Human IgG antibodies were cloned from single B lymphocytes sorted from pancreatic lymph node cells of three organ donors positive for islet autoantibodies, and from the peripheral blood of a patient with type 1 diabetes. Antibodies to insulinoma-associated antigen 2 (IA-2), GAD65, zinc transporter 8 (ZnT8) and Coxsackie B virus proteins were assayed by immunoprecipitation and by immunofluorescence on pancreatic sections.

Results Human IgG antibodies (863) were successfully cloned and produced from 4,092 single B cells from lymph

nodes and peripheral blood. Reactivity to the protein tyrosine phosphatase domain of the IA-2 autoantigen was detected in two cloned antibodies: one derived from a pancreatic lymph node and one from peripheral blood. Epitopes for these two antibodies were similar to each other and to those for circulating antibodies in type 1 diabetes. The remaining 861 antibodies were negative for reactivity to IA-2, GAD65 or ZnT8 by both assays tested. Reactivity to a Coxsackie viral protein 2 was detected in one antibody derived from a peripheral blood B cell, but not from lymph nodes.

Conclusions/interpretation We show evidence for the infrequent presence of autoantigen-specific IgG⁺ B lymphocytes in the pancreatic-draining lymph nodes of islet autoantibody-positive individuals.

Keywords Autoantibodies · Coxsackie virus · IgG expression · Single-cell PCR · Type 1 diabetes mellitus

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Abbreviations

BCR	B cell receptor
CDR3	Complementarity determining region 3
FLuc	Firefly luciferase
hAb	Human antibodies
HEK	Human embryonic kidney
HEV	Human enterovirus
IA-2	Insulinoma-associated antigen 2
JM	Juxtamembrane
LIPS	Luciferase immunoprecipitation system
PBMC	Peripheral blood mononuclear cells
PTP	Protein tyrosine phosphatase
RLuc	<i>Renilla</i> luciferase
VP	Viral protein
ZnT8	Zinc transporter 8

Introduction

Type 1 diabetes is associated with autoimmunity to islet cell proteins and with enteroviral infections in the inflamed pancreatic islet [1, 2]. Little, however, is known about the specificity or site of immune responses during pancreatic inflammation in type 1 diabetes. Immune responses are expected to occur within the diabetic pancreas [3], but molecular mimicry or immunisation at sites distant to the islets has also been suggested [4]. Few studies have examined lymphoid cells within the pancreas for evidence of autoreactive T and B cells. Insulin-reactive CD4⁺ T cells have been demonstrated in pancreatic lymph nodes of a patient with long-standing type 1 diabetes [5], proinsulin-reactive CD4⁺ T cells have been cloned from pancreatic islets of a patient [6], and islet-specific glucose-6-phosphatase catalytic subunit-related protein-specific CD8⁺ T cells have been observed within islets in pancreas sections of a patient with type 1 diabetes [7]. B lymphocytes are often found within islets [8], but the specificity of those in and around the pancreas is unknown. We had the opportunity to study pancreatic lymph nodes of organ donors who were islet autoantibody-positive and investigated whether the B cells within these were specific for antigens implicated in the pathogenesis of type 1 diabetes.

Methods

Donors and lymphocyte source

Lymph nodes were obtained from pancreases of organ donors in the Nordic Network for Islet Transplantation (Table 1). Sera from organ donors were tested for autoantibodies to GAD65 and to insulinoma-associated antigen 2 (IA-2) and, if positive, pancreases were processed and lymphoid tissue retrieved. Multiple pancreatic lymph nodes were isolated from each of three islet autoantibody-positive organ donors (PLN1, PLN2 and PLN3). Lymph node cells were dissociated, washed and frozen in serum/DMSO. Peripheral blood mononuclear cells (PBMC) were obtained from a patient (PBMC1) with type 1 diabetes at onset of disease. Collection of material was approved by local ethics review boards (Regional Ethics Committee in Uppsala: Dnr 2009/043 and Dnr 2009/371 [2012 12 16]; Bayerische Landesärztekammer in Munich). CD19⁺ B lymphocytes were isolated by magnetic bead isolation (Miltenyi Biotec, Bergisch Gladbach, Germany), stained with anti-CD19–phycoerythrin and anti-IgG–FITC (BD Biosciences, San Diego, CA, USA), and single live CD19⁺IgG⁺ lymphocytes sorted on a Becton Dickinson FACSaria II flow cytometer (Becton, Dickinson, Franklin Lakes, NJ, USA) into individual wells of PCR plates containing 10 µl TRIS+1 µl RNasin (Promega, Madison, WI, USA).

IgG production

Single-cell RT and a first PCR with constant region 3' primers and a mix of 4-, 3- and 7-signal peptide sequence primers covering all subgroups of heavy chain, kappa and lambda light chains, respectively (electronic supplementary material [ESM] Table 1), was performed directly on cells. A second nested PCR was used to amplify the entire variable and partial constant regions of the chains, and to N-terminally fuse these to the METDTLLLVLWVPGSTGD signal peptide. This was assembled into the remaining constant region, with cytomegalovirus promoter and polyadenylation signal, and transfected into human embryonic kidney (HEK) 293 T cells using PolyFect Reagent (Qiagen, Hilden, Germany). After 48 h, IgG concentration in supernatant fractions was measured by ELISA (ESM Fig. 1 and ESM Methods).

Antibody screening

Antigens of interest were N-terminally fused to Firefly luciferase (FLuc), *Renilla* luciferase (RLuc) or NanoLuc luciferase (Promega) and cloned into the pTNT vector (Promega). The following constructs were used to cover all known autoantibody epitopes of the antigens pTNTFLuc-IA-2 (amino acids 605–979), pTNTRLuc-GAD65 (96–585), pTNTFLuc-IA-2/zinc transporter 8 [ZnT8] WR (IA-2: 605–979; ZnT8W: 268–369, R325W; ZnT8R: 268–369), pTNTFLucZnT8W (268–369, R325W) and pTNTRLucZnT8R (268–369), as well as human enterovirus (HEV) fusion constructs as described (ESM Table 2). Fusion proteins were produced using SP6- or T7-based TNT-coupled rabbit reticulocyte system (Promega). For screening of antibody specificity, 10 µl clone supernatant fraction was incubated with 25 µl luciferase fusion protein (10⁷ relative light units [RLU]/well for FLuc or RLuc fusion proteins, 5×10⁶ RLU/well for NanoLuc and for HEV fusion proteins) in TRIS-buffered saline/Tween 20 and immune-precipitation assays performed (ESM Methods).

IA-2 epitope mapping of the human antibodies (hAb) was performed using constructs containing cytoplasmic IA-2 (amino acid 605–979), juxtamembrane (JM; 605–693), protein tyrosine phosphatase (PTP; 643–979) or central IA-2 PTP (643–937) regions, and alanine-substituted mutants of these, using radiobinding assays as previously described [9]. Inhibition by >50% was taken as evidence that amino acid substitution affected binding.

Immunofluorescence (ESM Methods) was performed on ready-to-use frozen monkey pancreas sections (Bio-Diagnostics, Upton-upon-Severn, UK).

Table 1 Cells and monoclonal antibodies produced and analysed, with description of samples used and characteristics of type 1 diabetic patients

B lymphocyte source	GAD65/IA-2 antibodies, U/ml	HbA _{1c} , % (mmol/mol)	HLA	IgG ⁺ CD19 ⁺ cells isolated, <i>n</i>	Successful heavy and light chain products, <i>n</i> (%)	Productive hAb, <i>n</i> (%)
PLN1 Female islet donor Age at sampling: 37 years Pancreatic lymph nodes	10/30	5.3 (34.4)	A3/31, B35/39, DR1/9	576	335 (58)	215 (37)
PLN2 Male islet donor Age at sampling: 63 years Pancreatic lymph nodes	13/64	5.2 (33.3)	A1/30, B18/60, DR1/3	768	196 (26)	105 (14)
PLN3 Male islet donor Age at sampling: 59 years Pancreatic lymph nodes	10,600/negative	5.8 (39.9)	A3/10, B7/12, DR3/15	1,488	459 (31)	261 (17.5)
PBMC1 Male donor with new-onset type 1 diabetes Age at sampling: 30.5 years (0.2 years after onset) PBMC	1,000/1,000	NA	HLA DRB1*03/*04 DQB1*02/*0302	1,260	536 (43)	282 (22)
Total				4,092	1,526 (37)	863 (21)

NA, not available

Results

Generation of hAb from single CD19⁺IgG⁺ B lymphocytes

A total of 2,832 CD19⁺IgG⁺ B lymphocytes were isolated from pancreatic lymph nodes and 1,260 from PBMC (Table 1). IgG heavy and light chain variable region pairs were amplified from 1,526 of 4,092 single cells (37% PCR efficiency, comparable to data given in [10]), and, after heavy and light chain assembly and transfection of HEK 293 T cells, IgG was detected in 863 (21% of isolated B cells) of the supernatant fractions.

Complementarity determining region 3 (CDR3) of the heavy and light chain PCR products from PLN1 and from PBMC1 were sequenced, and 571 heavy chain, 280 kappa light chain and 125 lambda light chain readable sequences were obtained (www.crt-dresden.de/research/crt-d-core-groups/bonifacio.html, accessed 1 June 2015; ESM Fig. 2). The same heavy or light chain sequence was not found in more than one cell from lymph nodes, but examples were seen in cells derived from peripheral blood (not shown).

Identification of islet autoantigen-specific hAb

The hAb were tested for their ability to bind the major autoantigens GAD65 and IA-2 and the two isoforms of ZnT8 (R and W). Four hAb showed increased binding to the IA-2/ZnT8R/W fusion protein (Fig. 1a), and two of these (PBMC1_A5 and PLN1_C11) were subsequently shown to

strongly bind IA-2-luciferase, but not ZnT8R- or ZnT8W-luciferase (Fig. 1b). No hAb bound GAD65-luciferase.

Both PBMC1_A5 and PLN1_C11 bound the PTP- and the central region constructs, indicating that their epitopes are included within amino acids 643–937 of IA-2 (Fig. 1c). Reactivity was further characterised using single amino acid mutants within the region 826–862, previously shown to encompass a major IA-2 epitope [9] (Fig. 1d). The binding profiles for the two hAb were almost identical and similar to that of existing hAb 96/3, despite different CDR3 amino acid sequences (PBMC1_A5: heavy chain CAKDSHVVAATPFDYW, light chain CQTWATGL#VF; PLN1_C11: heavy chain CAKEKWNFYDGSYSSGYDYW, light chain CQQAQKDFPITF; 96/3: heavy chain CARVLKGVATASFDWF, light chain CQVWDTRSDLVVF; ESM Table 3). Binding was markedly reduced by the mutations L831A, V834A, E836A, L839A, K857A, N858A, V859A or Q862A.

All hAb were also tested for reactivity to pancreatic islets, using immunofluorescence on sections of monkey pancreas. A strong islet-specific signal could be detected with the IA-2-binding PBMC1_A5 and with the control IA-2-specific hAb 96/3, but not with the IA-2-binding PLN1_C11 (Fig. 1e). None of the remaining hAb showed reactivity to monkey pancreas sections.

Reactivity of the hAb against HEV antigen

Type 1 diabetes is associated with Coxsackie B virus infection, with evidence of enteroviral proteins present in the inflamed islet [2]. We therefore examined whether any of the 863 hAb bound

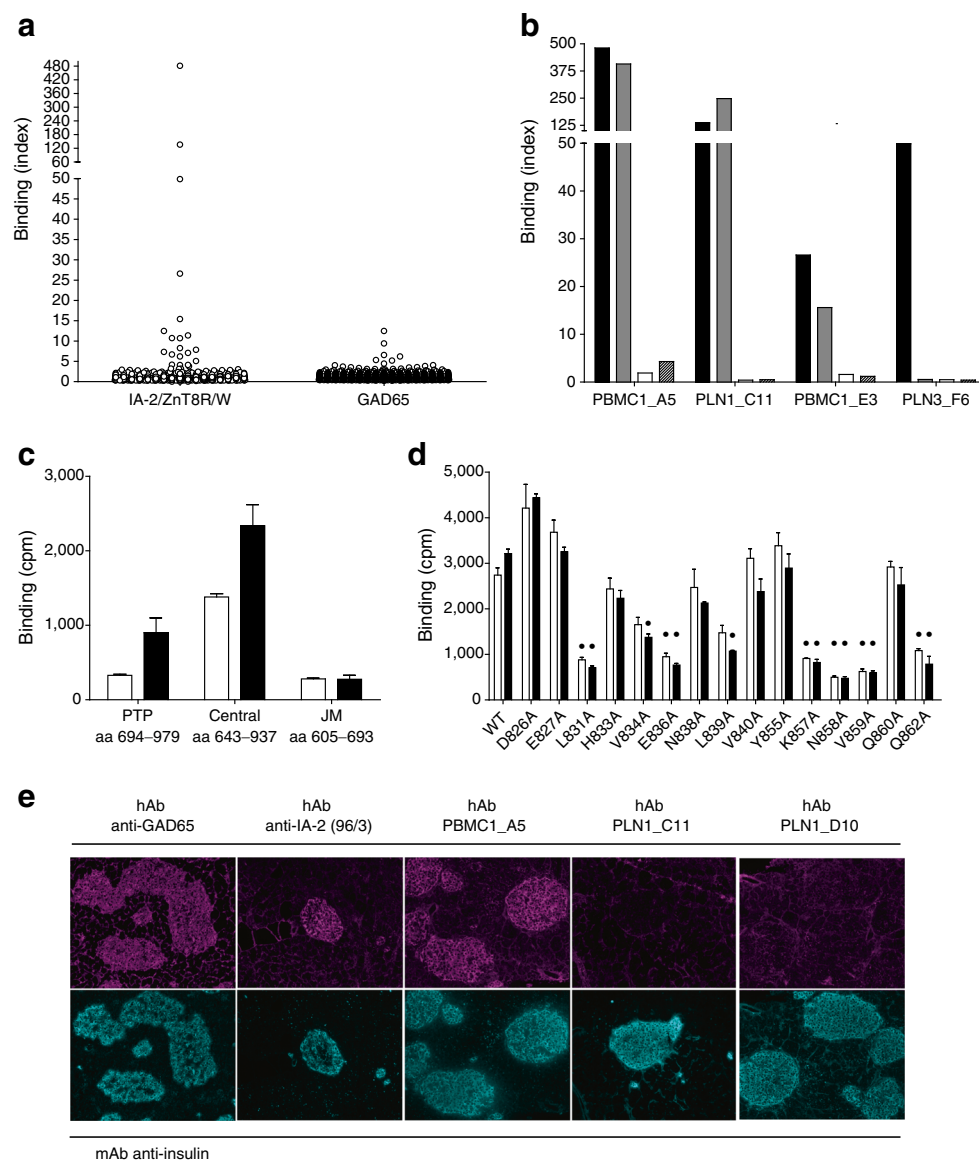


Fig. 1 (a) Immunoprecipitation assays for reactivity against IA-2, ZnT8 and GAD65. Dual-luciferase LIPS assays were performed on 863 hAb with FLuc fused to the IA-2 and ZnT8R/W antigens and RLuc fused to the GAD65 antigen. Binding was expressed as an index against the negative control. (b) Four hAb bound to the IA-2/ZnT8R/W fusion protein (PBMC1_A5, PLN1_C11, PBMC1_E3 and PLN3_F6) with an index >20, and were tested using single constructs containing IA-2, ZnT8R and ZnT8W. PBMC1_A5 and PLN1_C11 showed reactivity only for IA-2. PBMC1_E3 and PLN3_F6 did not bind any of these fusion proteins (index <20). None of the hAb were considered positive against GAD65 (a). Black bars, IA-2/ZnT8R/W; grey bars, IA-2; white bars, ZnT8R; striped bars, ZnT8W. (c) Mapping of the binding region of PBMC1_A5 (white bars) and PLN1_C11 (black bars). Using epitope mapping with

IA-2 deletion mutants (indicated on the x-axis), both hAb were shown to react with the IA-2 region 643–979 (PTP and central regions) in radiobinding assays. Binding is expressed as counts per minute (cpm). (d) Fine-mapping of the binding region of PBMC1_A5 and PLN1_C11. Single mutations in the central PTP region (aa 826 to aa 862, indicated in the x-axis) revealed that PBMC1_A5 and PLN1_C11 had comparable IA-2 amino acid requirements for binding. *Antibody binding <50% with respect to the wild-type protein. (e) Staining of monkey pancreatic sections with hAb (upper panels, magenta); staining with PBMC1_A5 and PLN1_C11, with anti-IA-2 (96/3) and GAD65 hAb as positive controls and a GAD-, IA-2- and ZnT8-negative antibody (PLN1_D10) as negative control, and co-staining for insulin (lower panels, cyan). aa, amino acid; mAb, monoclonal antibody

constructs representing the majority of the viral protein (VP) antigens found in Coxsackie B virus 1–6 using luciferase immunoprecipitation system (LIPS) assays (ESM Fig. 3). Only one hAb, PBMC1_A6, bound Coxsackie B virus protein, specifically the luciferase-CV-B1 VP2. Serum obtained from the same patient was positive against the same construct (not shown).

Discussion

We sought evidence for B cells against autoantigen and Coxsackie B virus in the lymph nodes of islet autoantibody-positive organ donors. One pancreatic lymph node-derived B cell was specific for IA-2 and therefore provided evidence for

B cell priming against autoantigen, but we found no evidence of B cells reactive to Coxsackie B virus in the pancreatic lymph nodes.

We had expected pancreatic lymph node B cells to be an enriched source of islet autoantibodies. However, B cells directed against pancreatic islet antigens were rare in the lymph nodes we studied. A previous study examining plasma cells in the cerebrospinal fluid of patients with multiple sclerosis also showed low frequencies of autoantigen-specific antibody-producing B cells in the vicinity of the lesions [11]. Unfortunately we could not investigate binding to one of the major islet autoantigens, insulin, due to high interference in the assays. We cannot exclude that the low frequency of islet antigen reactive B cells detected in our study was the consequence of sequence artefacts introduced during the cloning that affected the CDR3-binding region of the B cell receptors (BCRs). Nevertheless, the study produced two antibodies against IA-2, one from a lymph node and one from peripheral blood, both to the PTP region, with binding profiles similar to each other and to other type 1 diabetes-derived autoantibodies [9]. This epitope, therefore, is a common target of autoreactive B cells in type 1 diabetes. Subtle differences in the fine specificity of the two IA-2 autoantibodies were shown on the basis of reactivity with mutated IA-2 constructs and heterogeneous reactivity with islets on monkey pancreas. Absent reactivity on monkey pancreas for one antibody may be due to an amino acid difference at position 862 between human and macaque IA-2.

Because of reports of HEV infection in the type 1 diabetic pancreas [2], we looked for evidence of pancreatic lymph node B cells directed to Coxsackie proteins. Although we were able to identify one Coxsackie B virus-specific BCR from peripheral blood B cells, we did not see binding from the lymph node-derived BCRs. This may either indicate that the patients had little or no exposure to Coxsackie B virus in the pancreas or that our methods were inadequate to faithfully produce hAb.

In summary, we provide evidence that there are IgG⁺ B lymphocytes primed against islet autoantigen at low frequency in lymph nodes that drain the pancreas of islet autoantibody-positive individuals.

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Compliance with ethical standards

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